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Sex and Ontogenetic Variation in the Crest of *Numida meleagris*: Implications for Crested Vertebrates

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ABSTRACT

Crested vertebrates are known from a wide variety of modern and fossil taxa, however, the actual formation and function of the crest is still debatable. Among modern birds, the globally distributed guinea fowl (*Numida meleagris*) is characterized by having a cranial bony crest (overlain by keratin), but surprisingly little is known about its development. Here, we studied the crest of 202 wild guinea fowl from the same population, using anatomical measurements as well as 2D-morphometry. Our results show that juveniles have smaller skulls than adults and have smaller, simpler crests that are visible even in very young individuals. Among adults, female skulls are smaller than males, and they have smaller, simpler shaped crests, which permit a discrimination between the sexes of 93% when the keratin is preserved with the bony crest, and of 89% when only the bony crest is available. By extrapolation, these results confirm that the crest can be used as an ontogenetic character, as well as for sex discrimination in the fossil record. Our results also show that the overlying keratin does not always mimic the underlying bony crest, which should be considered when reconstructing extinct crested vertebrates. Anat Rec, 303:1018–1034, 2020. © 2019 American Association for Anatomy

Key words: helmeted Guinea fowl; 2D-morphometric; birds; cranial crest

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Many modern and fossil vertebrates have a crest positioned dorsally on the skull, which varies in terms of size, structure, shape, and functions. Some crests are membraneous (Barsbold, 1988; Frey et al., 2003), but by far the majority is composed of bone and is overlain by keratin (Naish and Perron, 2016). Cranial crests have been linked to various ecological adaptations, including muscle attachment (Measey et al., 2009), thermal regulation (Kellner and Campos, 2002; Eastick et al., 2019), sexual dimorphism (Bennett, 1992), species recognition (Horner et al., 2000), as an acoustic tool (Farke et al., 2013), and as a feeding adaptation (Wang et al., 2014b). Although cranial bony crests are known for a large number of vertebrates, including dinosaurs (Dodson, 1975;

Additional Supporting Information may be found in the online version of this article.

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Welles, 1984; Clark et al., 2002; Hone et al., 2012; Farke et al., 2013; Lü et al., 2017; Funston et al., 2018), pterosaurs (Bennett, 1992; Bennett, 1993; Bennett, 1996; Bennett, 2002; Kellner and Campos, 2002; Hone et al., 2012; Wang et al., 2014a; Wang et al., 2014b), and birds (Naish and Perron, 2016), they have been surprisingly scantily studied and they are therefore poorly understood, even for modern birds, thus making interpretations of such fossilized structures difficult.

Among modern birds, many groups sport a distinctive crest of feathers on their heads, which is mainly utilized for sexual selection. Several groups of birds show a bony crest on their heads (e.g. Hornbills), but this crest is mostly linked to their beak rather than their cranium. Only a few groups have a bony crest on their cranium including guinea fowls (family Numididae), megapolid (*Macrocephalon*) and cassowaries (genus *Casuarius*) (Mayr, 2018). In these cases, although they are covered with keratin, no membrane is attached to the crest, as deduced for some pterosaurs (Hone et al., 2012), and they are also not used for muscular insertions as in some chameleons (Measey et al., 2009). Thus, the function of the cranial crest remains enigmatic. In the case of the cassowaries, several hypotheses have been proposed to explain the function of this crest: a sexual ornamentation, a weapon used in intraspecific combats, a tool for moving foliage and detritus on the rainforest floor or for knocking hanging branches to dislodge fruit, a cranial protection when charging through undergrowth, a resonance box for low frequency communication [see Naish and Perron, 2016 for a summary] or as a thermoregulation tool (Eastick et al., 2019). Until now, only a few studies have been published about the crest of cassowaries (Parker, 1866; Marshall, 1872; Dodson, 1975; Crome and Moore, 1988; Richardson, 1991; Mack and Jones, 2003; Naish and Perron, 2016; Eastick et al., 2019), and even more surprisingly, only one preliminary paper has dealt with the crest of the guinea fowl (Prinsloo et al., 2005) and attempted to use anatomical measurements to deduce sex differences. The results of these earlier studies are generally inconclusive and raise the need for further studies in this area, as highlighted recently by Mayr (2018).

Guinea fowls belong to the Numididae family in the Galliformes order. They are endemic to Africa and have been introduced widely elsewhere. Four genera are known among Numididae, including the genus *Numida* in which only one species, *Numida meleagris*, the helmeted guinea fowl is described. The latter has a small-unfeathered head and a round body covered with a gray-black plumage spangled with white. These omnivorous birds have life spans of about 12 years and they are mostly terrestrial and gregarious, living in flocks of about 25 birds. Nine subspecies of *Numida meleagris* are recognized based on morphological features such as the ceres (wart-like cartilaginous structure at the dorsal junction between the beak and the skull), gape wattle shape-color, "face" color, feathering on the hind-neck and, especially, the size, shape, and color of its keratin-covered helmet (Crowe, 1978; Martínez and Kirwan, 2017). The subspecie studied here, *Numida meleagris coronate* (Gurney, 1868), is known in Zimbabwe, eastern Botswana, and South Africa with populations south of the Orange River and east of Port Elizabeth introduced by humans. They have long hook-sabre-shaped horn-colored helmet, blue faces, blue pennant-shape wattles with red tips, bare cere, and long filoplume feathers confined to the mid-hind-neck.

This study focuses on the crest of 202 skulls of the helmeted guinea fowl (*Numida meleagris coronate*). It provides, for the first time, an understanding of the 3D anatomical development of this structure, documents the variability of the crest through the ontogeny and between the sexes, and suggests its possible function. The insight obtained regarding the crest of this modern bird is pertinent for extrapolation to crested fossil vertebrates.

MATERIAL

This study is based on an initial large sample of 309 heads of the helmeted guinea fowl (*Numida meleagris coronatus*), all housed in the comparative osteology collection of the Department of Biological Sciences, at the University of Cape Town (South Africa). All the specimens were collected in Bloemfontein, in the central part of South Africa, during recreational hunting activity, and the skulls were donated for scientific studies. The hunting origin of the samples may have introduced a bias in the size of the individuals, as only the "larger" individuals were killed. Initially, all the soft tissues were preserved, including muscles, skin, feathers and the keratin part of the beak and the crest. The specimens were identified as juveniles, adult males, and adult females using the size of the skull, the size and the color of the crest, and the size and the color of the wattles, as defined previously by Prinsloo et al. (2005) for the skull, crest, and wattles and Abdul-Rahman et al. (2015) for the wattles. According to these earlier studies, adults are larger than juveniles, have larger crests and show two long wattles. Among the adults, males have generally larger skulls, and they have larger crests and wattles than females (Fig. 1). Following these external observations, 119 juveniles and 190 adults were identified, with 76 of them being females and 114 males.

All the skulls were left above the ground to skeletonize naturally. After 6 months, the skulls were collected, and they were cleaned using water, soap, and a toothbrush to remove all the residuals of feathers and skin. For some specimens, the keratin of the bony crest and the tip of the beak were removed and kept associated with the specimens, permitting the study of the specimens with and without their keratin parts.

After the skulls were cleaned, the juveniles were secondarily validated using the fused/unfused state of the suture between the two frontal bones (which are the last bones to fuse among juvenile birds) that form the bony crest (Hogg, 1978). Thus, we identified juveniles as ones having a crest with the two frontal bones incompletely fused or with the trace of the suture still visible. Using these criteria, 26 previously identified juveniles were reassessed as adults, although we were unable to identify the sex of the specimens since the telling soft tissues were already removed during the initial preparation. For this reason, these adults are identified as "adults *indet*." in the consequent aspects of this research and will be used only when the sex identification is not required.

Furthermore, although we initially had 309 specimens, we used only the ones that preserved the keratin cap and the bony crest, as well as the ones for which the crest and the skull are complete. After applying this filter, our sample size was reduced to 202 specimens, including 69 juveniles and 133 adults, with 64 males and 43 females, as well as 26 adults *indet*. Depending on the measurements and information required for each analysis, we used a

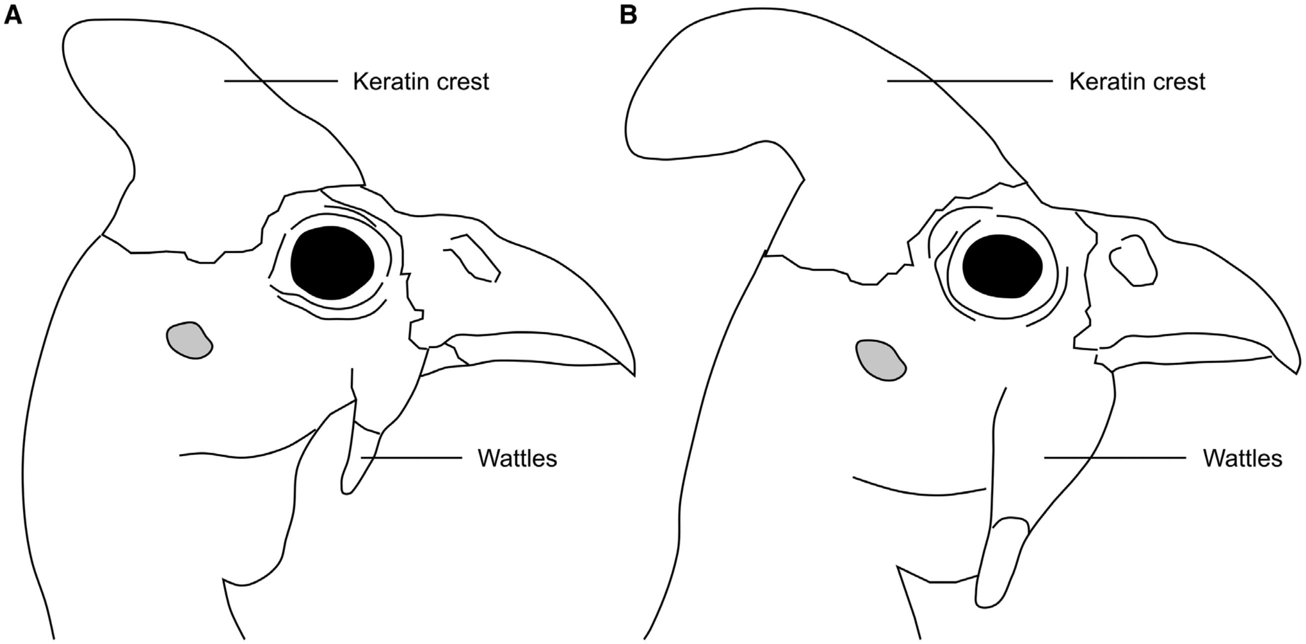

Fig. 1. Anatomical characters used to distinguish adults' males and females before skeletonization. (**A**) Adult female, (**B**) Adult male.

different subset of this large data set for sections of the study (Table 1).

METHODS

Anatomical Measurements

The anatomical terms utilized in this study are *sensu* Baumel et al. (1993). All the skulls were photographed in dorsal, ventral, lateral, cranial, and frontal views, and measurements were made using a digital caliper (Fig. 2). The length, height, and width of the bony crest were measured in millimeters for all the specimens for which the keratin cap could be removed. The Bcl was measured between the most anterior part of the suture of the two frontals and the most dorsal part of the crest (Fig. 2). The bony crest height (Bch) was measured between the most ventral part of the *processus postorbitalis* and the most dorsal part of the bony crest (Fig. 2). The bony crest width was measured at the base of the crest, in cranial view (Fig. 2). The skull length (Sl) was measured between the most anterior and the most posterior part of the skull in lateral view, for the specimens for which the keratin cover at the tip of the beak was removed (Fig. 2). The skull width was measured in the cranial view, between the two *processus postorbitalis*, in the most ventral part (Fig. 2).

We used the Shapiro test (Shapiro and Wilk, 1965) to check the normality of the data for each measure among the juveniles, the adult males, and the adult females. Because the measurements do not follow a normal distribution, nonparametric statistical analyses were performed using the software PAST 3.13 (Hammer et al., 2001) to compare the different measurements. The difference of the distribution for the different measures between juveniles and adults, and adult males and adult females, was completed using the nonparametric Mann–Whitney *U* test (Mann and Whitney, 1947). To this end, all the measurements of the skull and the crest for all the juveniles and the adults were used (Tables 1, 3, and 4). The results of this test between juveniles and adults provide the statistic U (%), which is calculated using the U value and the $U_{\max}$ (=$n_{\text{juveniles}} \times n_{\text{adults}}$) and corresponds to the percentage of randomly chosen juveniles that will be smaller than a randomly chosen adult. A similar approach was used to study the difference between adult males and adult females. Using this value, we can, therefore, determine the best parameter to discriminate between juveniles and adults, as well as between adult males and adult females.

Because the shape of the crest is highly variable, two specimens with the same linear measurements of the crest can have a crest that has quite different shapes. We, therefore, measured the crest area in the lateral view. To standardize the photos for all the specimens, all the lateral images were taken with the two *processus postorbitalis* aligned, as well as the two quadrates (Fig. 3, all the pictures in lateral view used for this work are available in the following DOI: 10.5523/bris.x4rzhi87ptab2x1pk4aqhbiac, which can be open with https://dx.doi.org/). On these images, the anterior and posterior bases of the crest were located using two landmarks (LM), respectively L7 and L8. The shape of the crest was drawn using 30 points (semi-landmarks = semi-LM) homogeneously distributed and manually positioned to outline the external shape of the crest (see section 2D Geometric Morphometrics for more details; Fig. 3). These two steps were done using the Thin Plate Spline (TPS, Bookstein, 1989) tools [tpsUtil version 1.64; Rohlf (2015a) and tpsDig2 version 2.22; Rohlf (2015b)] and applied on 171 specimens for the bony crest and 119 specimens for the keratin crest (Table 1). We measured the area of the crest as the area below the semi-LM and closed by the link between L7 and L8 (Fig. 3). To do so we used the composite trapezoidal rule as implemented in scipy version 1.1.0 (Jones et al., 2001, Supplementary Program 1). The position of the points (semi-LM and LM) and the scale identification on the pictures were done

TABLE 1. Summary of the material used for each analysis

	Anatomical measurements			2D morphometry	
	Comparison juvenile versus adults	Comparison adults males versus females	Bony crest area versus Keratin crest area	PCA bony crest shape juveniles versus adults	PCA keratin crest shape adults male versus females
Bcw	202 specimens (69 juv., 133 adults)	107 specimens (64 ♂, 43 ♀)	/	/	/
Bcl	202 specimens (69 juv., 133 adults)	107 specimens (64 ♂, 43 ♀)	/	/	/
Bch	200 specimens (69 juv., 131 adults)	105 specimens (62 ♂, 43 ♀)	/	/	/
Bca	177 specimens (53 juv., 124 adults)	100 specimens (61 ♂, 39 ♀)	94 specimens (60 ♂, 34 ♀)	52 juv., 119 adults (61 ♂, 35 ♀, 23 *indet.*)	119 specimens (61 ♂, 35 ♀, 23 *indet.*)
Kca	104 specimens (4 juv., 100 adults)	95 specimens (61 ♂, 34 ♀)	94 specimens (60 ♂, 34 ♀)	/	100 specimens (61 ♂, 34 ♀, 5 *indet.*)
BcCS	177 specimens (53 juv., 124 adults)	100 specimens (61 ♂, 39 ♀)	/	/	/
KcCS	104 specimens (4 juv., 100 adults)	95 specimens (61 ♂, 34 ♀)	/	/	/
Sw	194 specimens (65 juv., 129 adults)	103 specimens (60 ♂, 43 ♀)	/	/	/
Sl	142 specimens (36 juv., 106 adults)	105 specimens (62 ♂, 43 ♀)	/	/	/
IS skull	130 specimens (31 juv., 55 ♂, 30 ♀, 14 *indet.*)	85 specimens (55 ♂, 30 ♀)	/	/	/
IS bony crest	130 specimens (31 juv., 55 ♂, 30 ♀, 14 *indet.*)	85 specimens (55 ♂, 30 ♀)	/	/	/

♂: males, ♀: females, juv.: juveniles.
Bcl: Bony crest length, Bch: Bony crest height, Bcw: Bony crest width, Bca: Bony crest area, Kca: Keratin crest area, BcCS: Bony crest centroid size, KcCS: Keratin crest centroid size, Sl: Skull length, Sw: Skull width, IS skull: Isometric size of the skull, IS bony crest: Isometric size of the bony crest. The list of each specimen used for each measurement and analysis can be found in Supplementary Table 1.

manually, which could include a human sampling error. To estimate the importance of this error, and if necessary provide an error correction, six rounds of measures were done for five skulls. We obtained a mean error of 3.36% for the crest area estimation, which is small enough to consider our results relevant without a secondary correction. In parallel, using TPS tools and the same lateral pictures, the centroid size was measured, corresponding to the square root of the sum of squared distances of a set of LM or semi-LM from their centroid (center of gravity) (Fig. 3).

These measures were used to compare juveniles and adults, using all the specimens complete enough to permit the measurements of the length, height, width, area and centroid size of the bony crest, and the length and the width of the skull (number of specimens summarized in Table 1; Supplementary Table 1).

To further explore the variability of the bony crest, some of these skulls were used to assess if there is an allometric relationship, corresponding to a change of shape linked with size (Klingenberg, 2016), between the growth of the crest and the growth of the skulls (used as a proxy of the body size). To this end, we used all the specimens for which the crest and the skull were complete enough to permit the measurements of the length, height, width, and area of the bony crest, and the length and the width of the skull. Since the specimens were killed during hunting, we do not know their age at death. As a proxy for the growth of the juveniles and their ontogenetic stages, the isometric size of the skulls was calculated for each individual. The isometric size of the crest, corresponding to the mean value of the logarithm (Log10) of the length, height, width, and area of the bony crest was calculated, as well as the isometric size of the skull, corresponding to the average logarithm of the length and the width of the skull (Mosimann, 1970). The keratin area was not used because only four juvenile specimens were preserved with the keratin cap (Table 1; Supplementary Table 1). To highlight the potential correlations between the isometric size of the bony crest and the isometric size of the skull for juveniles, adult males, and adult females, linear regressions, as well as Spearman R correlation test, were calculated.

The variability among the adult guinea fowl was studied using all the specimens showing fused frontals and for which the crest and the skull were complete enough to permit the measurements of length, height, width, area and centroid size of the bony crest, area and centroid size of the keratin crest, as well as the length and the width of the skull (number of specimens summarized in the Table 1; Supplementary Table 1). Finally, the difference between the bony crest and the keratin crest that covers the bone was calculated using all the adult specimens for which the keratin part was present but removed from the bone during the preparation and kept with the specimen (number of specimens summarized in Table 1; Supplementary Table 1).

2D Geometric Morphometrics

Independent of the size of the crest or the skull, the crest shape of the guinea fowl is highly variable. This variability was studied using a 2D geometric morphometric approach (Zelditch et al., 2012), based on the utilization of coordinates of LM and semi-LM following the work of Bookstein (1997). This approach makes it possible to

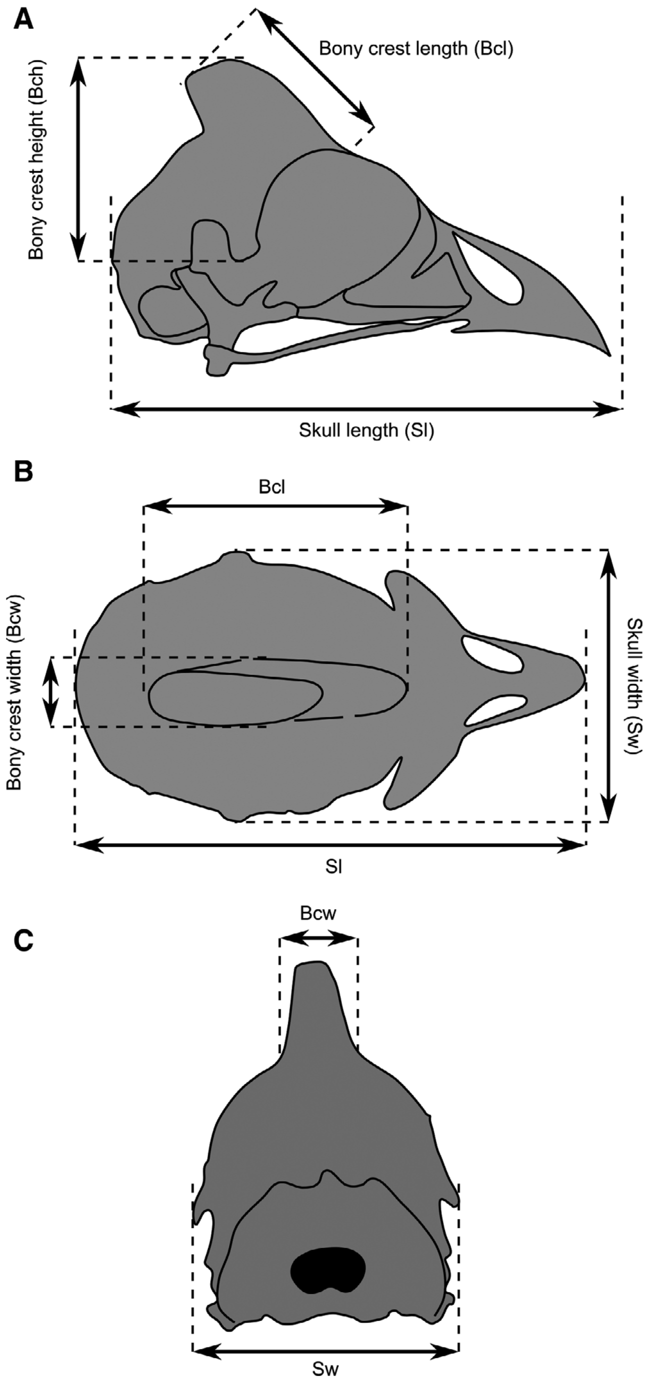

Fig. 2. Location of the anatomical measurements made on the guinea fowl skulls. (**A**) Lateral view, (**B**) dorsal view, and (**C**) Cranial view.

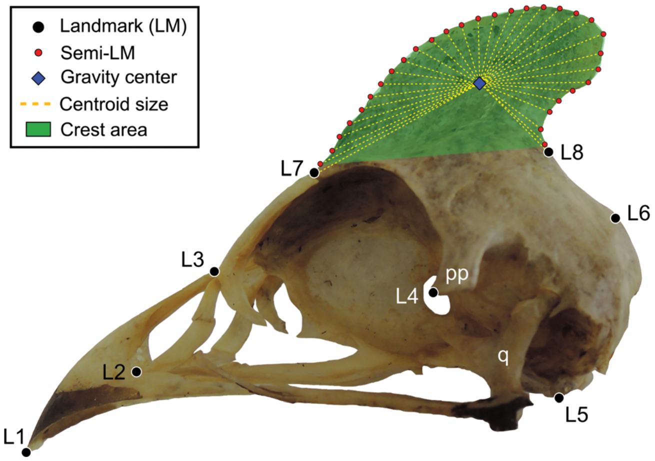

Fig. 3. Location of the morphometric points and measurements of the bony crest. Black dots: 8 landmarks (LM); red dots: the 30 semi-landmarks, which represented the shape of the crest; green area: crest area; blue diamond: gravity center; yellow lines: links between the gravity center and the LMs and semi-LMs, the square root of the sum of the squared of these links corresponds to the centroid size; pp: *processus postorbitalis*, q: quadrates.

describe precisely areas without anatomical LM but with a strong biological interest, in this case, the crest. Semi-LM are allowed to slide along their curve minimizing their position in relation to the average shape of the entire sample.

To this end, we used the same lateral 2D-images of the skulls that were used to measure the crest area, and we define eight LM on specific homologous positions on the skull (Fig. 3). The LM were precisely defined (Table 2) and positioned on the pictures using TPS tools (tps.Dig2). Because we cannot define any homologous landmark on the crest, we used the concept of semi-LM as defined by Bookstein (1991). Here, we used 30 semi-LM, equidistantly spaced along the external border of the crest, between the LM 7 and LM 8. These are the same semi-LM we used to measure the crest area (Fig. 3). Semi-LM on each specimen can slide along the crest curve to minimize the bending energy between this individual and the Procrustes mean shape (Bookstein, 1997). This criterion was chosen because it acts on all points while distinguishing anatomical LM from semi-LM, unlike the procrustes criterion where each semi-LM slide separately (Gunz and Mitteroecker, 2013). Then, a General

TABLE 2. Explanations of the exact location of the eight landmarks (=LM)

Landmark number	Anatomical description of the location of the point
LM1	Tip of the beak
LM2	Point of convergence between the ventral part of the *os nasale* and the *os premaxillare*
LM3	Point of convergence between the dorsal part of the *os nasale* and *os lacrimale*
LM4	Most anterior and ventral part of the *processus postorbitalis*
LM5	Point in the most ventral part of the *processus paroccipialis*
LM6	Point in the most dorsal part of the *processus cerebelli*
LM7	Point at the base of the crest in the anterior part
LM8	Point at the base of the crest in the posterior part

Procrustes Analyses (Rohlf and Slice, 1990) was performed as well as a Principal Component Analyze (PCA) on procrustes residuals permitting the quantification and the visualization using TPS of the different shape of the crest along the different axes (Klingenkerg, 2013). This method was applied to the crest shape variability in two cases: between juveniles and adults, and between adult males and adult females. In the first case, we used all the specimens with a complete skull and the bony crest preserved. Unfortunately, the shape variability could not be tested for the keratin crest because only four juveniles were available with the keratin preserved (Table 1; Supplementary Table 1). In the case of the variability of the crest shape among adults, we used all the specimens with the two frontals fused, a complete skull, and the bony crest and the keratin crest preserved (Table 1; Supplementary Table 1).

RESULTS

Juvenile Stage

General observations. Among our sample, 69 specimens are juveniles, showing different stages of maturity and differences in terms of the size and shape of the bony crest. The bony crest is present in all the juvenile specimens, even for the youngest in which the frontals–parietals sutures are unfused (e.g., specimens 100, 194, and 195) (Fig. 4A–C). This crest is formed dorsally from the two frontal bones, following the anteroposterior direction, and presents an external surface that is smooth and even (Fig. 4). Despite not having the precise age of the specimens at death, the youngest specimen seems to be specimen 194 because it shows an incomplete suture between the parietal and the squamosal bones and it is the smallest for most of the measurements (Fig. 4A–C, Supplementary Table 1). On the other hand, the most mature juvenile (specimen 187; Fig. 4D–F) shows the parietals completely fused with the frontals, and the suture between the two frontals is almost completely fused but it is still visible, permitting its unambiguous identification as a juvenile. This specimen has a large and well-developed bony crest and has the largest dimensions among the juveniles for most of the measurements.

Juveniles–adults distinction. The comparison between juveniles and adults shows that for all the

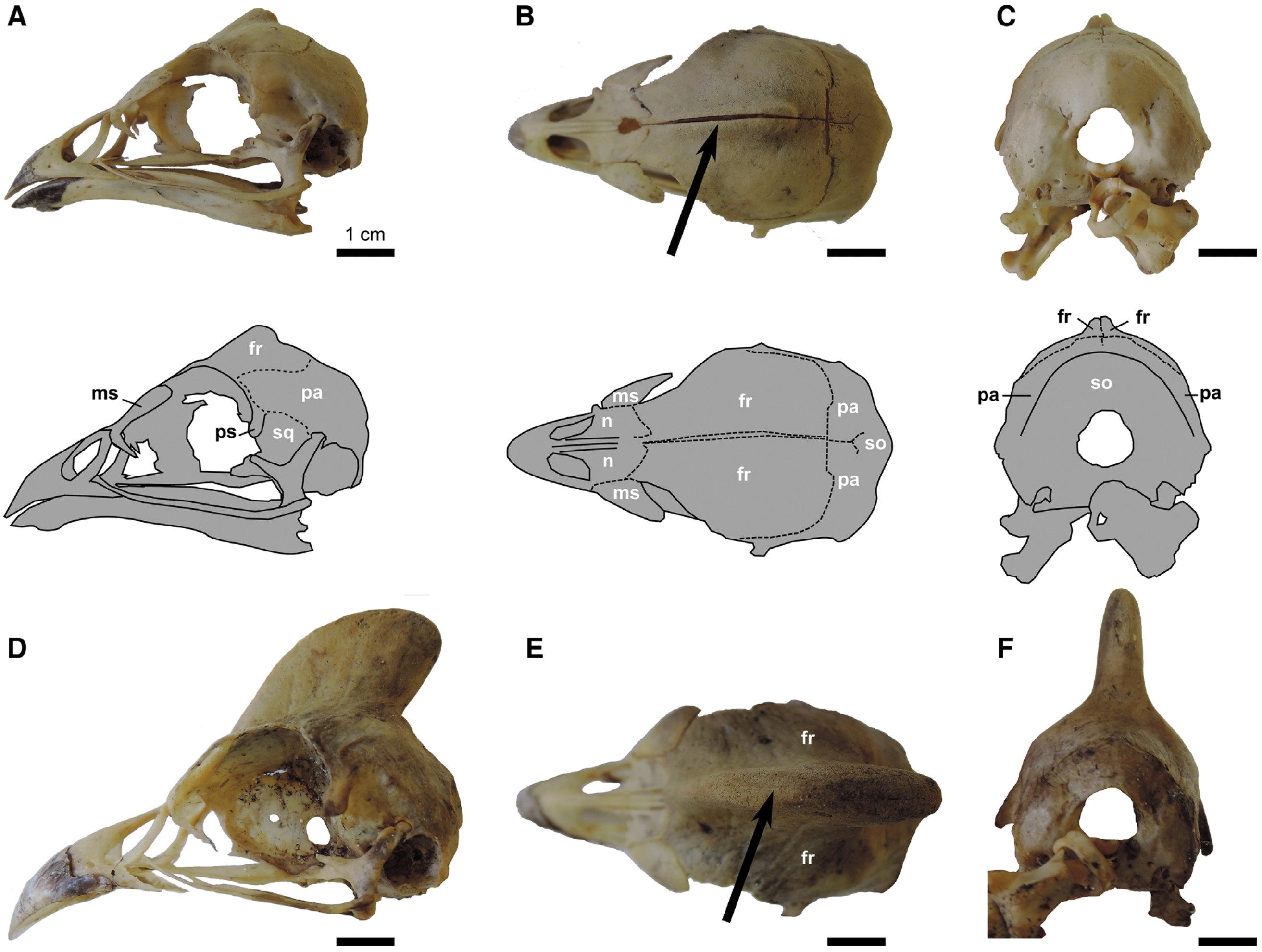

Fig. 4. Variability of the bony crest of the juveniles. Specimen 194: (**A**) picture and interpretative drawing in lateral view, (**B**) picture and interpretative drawing in dorsal view, and (**C**) picture and interpretative drawing in cranial view. Specimen 187: (**D**) picture in lateral view, (**E**) picture in dorsal view, and (**F**) picture in cranial view. fr: frontal, ms: mesethmoid, n: nasal, pa: parietal, so: supraoccipital, sq: squamosal.

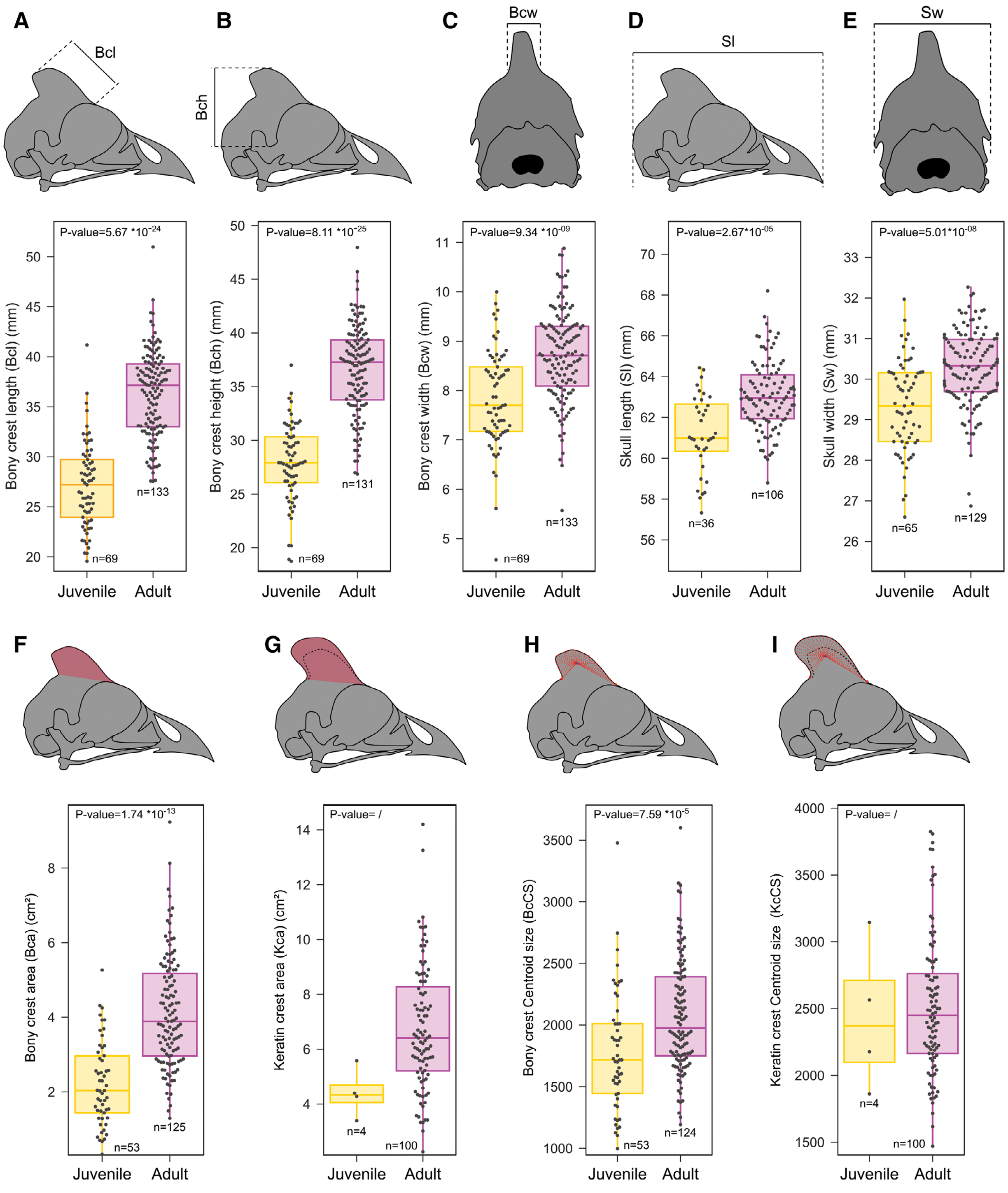

Fig. 5. Variability of the anatomical measurements of the crest and the skull of the guinea fowl for juveniles and adults. (**A**) Variation in the bony crest length, (**B**) variation in the bony crest height, (**C**) variation in the bony crest width, (**D**) variation in the skull length, (**E**) variation in the skull width, (**F**) variation in the bony crest area, (**G**) variation in the keratin crest area, (**H**) variation in the bony crest centroid size, and (**I**) variation in the keratin crest centroid size. *n* = number of specimens; *P*-value: result of the nonparametric Mann–Whitney *U* statistic test.

TABLE 3. Summary of the statistical Wilcoxon–Mann–Whitney tests, to compare the distribution between adults and juveniles

	Bcl	Bch	Bcw	Bca	Kca	BcCS	KcCS	Sl	Sw
n (adults)	133	131	133	124	100	124	100	106	129
n (juveniles)	69	69	69	53	4	53	4	36	65
P-value	$5.67E^{-24}$	$8.11E^{-25}$	$9.34E^{-09}$	$1.77E^{-13}$	0.018	$7.59E^{-0.5}$	0.77	$3.25E^{-06}$	$5.01E^{-08}$
U	609.5	516.5	2,325.5	986	60	1875	182	915	2,180
U_{max}	9.177	9.039	9.177	6.572	400	6.076	400	3.816	8.385
U (%)	93	94	75	85	85	69	55	76	74

n: number of specimens, *U*: value of the statistic, U_{max} = *n*(adults) × *n*(juveniles), *U*(%) = 100 − (U/U_{max} × 100). Bcl: Bony crest length, Bch: Bony crest height, Bcw: Bony crest width, Bca: Bony crest area, Kca: Keratin crest area, BcCS: Bony crest centroid size, KcCS: Keratin crest centroid size, Sl: Skull length, Sw: Skull width.

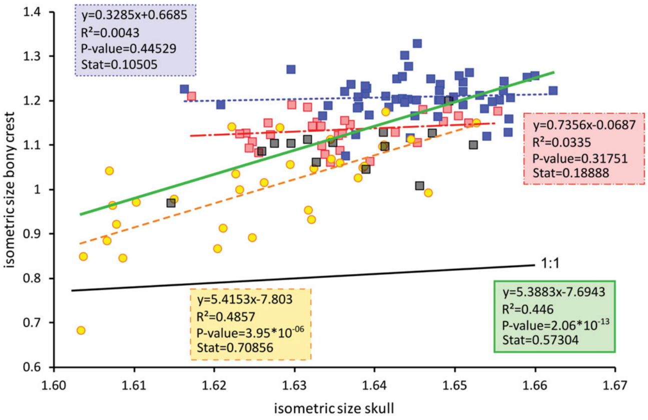

Fig. 6. Variation of the log of the isometric size of the skull and the log of the isometric size of the bony crest. Yellow dots: juveniles, red squares: adult females, blue squares: adult males and black squares: adults *indet*.. – – –: linear regression for the juveniles, with the equation of the regression and the R^2 in the yellow box, –·–·–: linear regression for the adult females, with the equation of the regression and the R^2 in the red box, ---: linear regression for the adult males, with the equation of the regression and the R^2 in the blue box, and –: linear regression for all the specimens, with the equation of the regression and the R^2 in the green box. *P*-value and statistics in the boxes are the results of the Spearman correlation test.

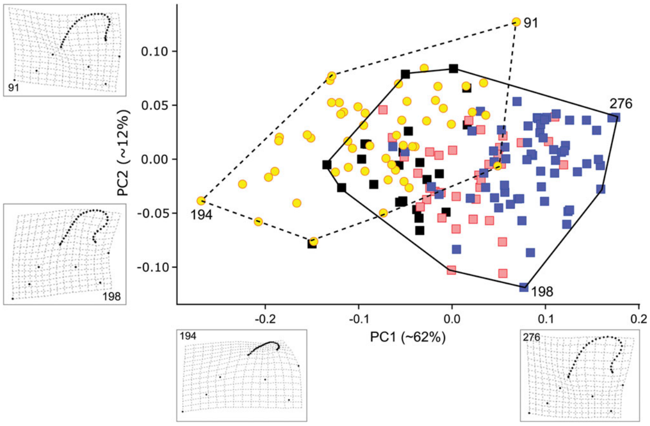

Fig. 7. PCA obtained using the bony crest of juveniles and adults. Yellow dots: juveniles, blue squares: adult males, red squares: adult females, and black squares: adults *indet*.

parameters measured, the juveniles are always smaller than the adults (Fig. 5), which has been confirmed by the statistical analysis (see in Table 3 that the *P*-value is always below 0.05; except for keratin centroid size (KcCS) for which only four juvenile specimens were available). Our results show that the statistic *U* is not the same for all the parameters. Indeed, *U* is equal to 94% for the bony crest height (Bch) and 93% for the bony crest length (Bcl), which means that for these two measurements, a very limited percentage of juveniles chosen randomly will be larger than an adult chosen randomly (respectively 6% and 7%). Therefore, Bch and Bcl are two good parameters to distinguish juveniles from adults. Contrarily, the bony crest centroid size does not show a significant difference between juveniles and adults, with *U* equal to 69%. The keratin crest area (Kca) and the keratin centroid size (KcCS) should not be taken into consideration, because only four juveniles specimens were used, which all corresponded to late-stage juveniles with large skulls and well-developed crests, making the results difficult to interpret (Fig. 5G,I).

Variability among the juveniles. Among the juveniles, the bony crest presents an important variable for anatomical measurements as well as for the assessment of its shape. Figure 6 shows a positive allometric relationship between the isometric size of the skull and the isometric size of the bony crest. The correlation statistical test Spearman *R* applied on our data set shows that the larger the skull, the larger is the bony crest (Spearman correlation coefficient = 0.70856). The slope of the linear regression for the juveniles is 5.4153, which means that for the juveniles, the bony crest grows faster than the skull.

This high variability of the simple measurements of the bony crest size is also visible for the shape of the bony crest as shown in Figure 7. This PCA, which includes all the adults and the juveniles with a complete bony crest and a complete skull, shows that the shape of the juveniles is different from that of the adults. Even if there is a significant overlap between the juveniles and the adults, the first principal component (PC1) explains 62% of the variability and indicates that the juveniles have a bony crest more compact and compressed dorsoventrally, while the adults have a bony crest well developed, with a significant extension along the dorsoventral axis.

It is worth noting that the juveniles overlap mainly with female adults and the adults *indet*. Indeed, the juveniles show a large variability in their bony crests with some having short, compact crests to others having well = developed crests, like those observed among the

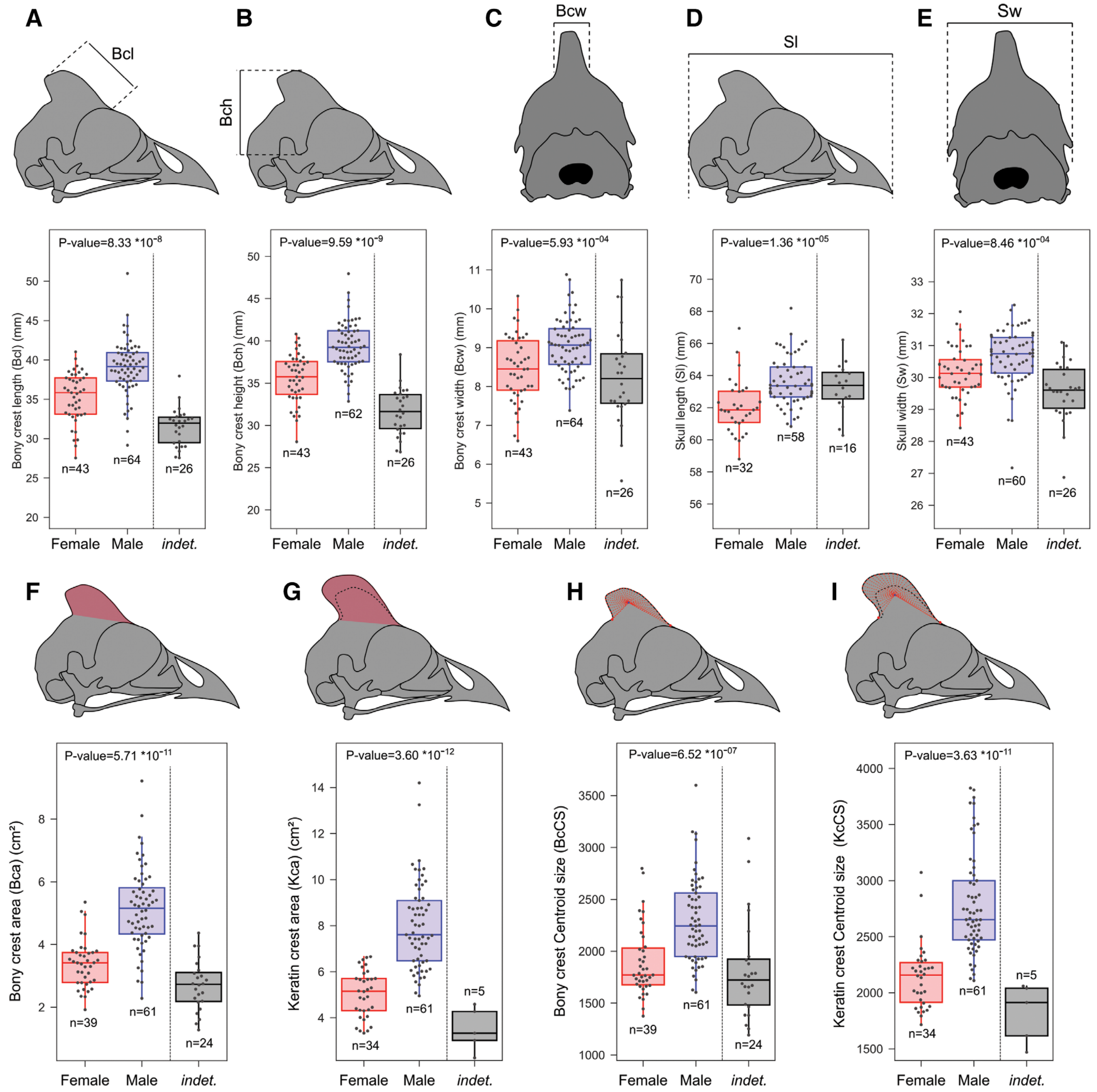

Fig. 8. Variation of the anatomical measurements of the crest and the skull of the guinea fowl for the adult males, adult females, and adults *indet*. (**A**) Variation in the bony crest length, (**B**) variation in the bony crest height, (**C**) variation in the bony crest width variability, (**D**) variation in the skull length, (**E**) variation in the skull width, (**F**) variation in the bony crest area, (**G**) variation in the keratin crest area, (**H**) variation in the bony crest centroid size, and (**I**) variation in the keratin crest centroid size. *n* = number of specimens; *P*-value: result of the nonparametric Mann–Whitney *U* statistic test.

adults. However, the bony crest shape appears to be a relatively good parameter to distinguish between juveniles and adult males.

Adults Stages

Variability among the adults. The bony crest surface of the adults is rough and porous, which contrasts with the smooth surfaces exhibited by the juveniles. Among the adults, the distinction between adult males and females in our sample of helmeted guinea fowl was initially done using external characteristics. In order to avoid a circular approach, using the same parameters to initially distinguish the adult's groups (males and females) and to infer the variability and the differences between these same groups, we secondarily validated our

TABLE 4. Summary of the statistical Wilcoxon–Mann–Whitney tests, to compare the distribution between adult males, adult females, and adults *indet*

	Bcl	Bch	Bcw	Bca	Kca	BcCS	KcCS	Sl	Sw
n (males)	64	62	64	61	61	61	61	58	60
n (females)	43	43	43	39	34	39	34	32	43
P-value	8.33E^{-08}	9.59E^{-09}	5.93E^{-04}	5.71E^{-11}	3.60E^{-12}	6.52E^{-07}	3.63E^{-11}	1.36E^{-05}	8.84E^{-04}
U	532	452	835	262	141	485	184	411.5	790.5
$U_{\max}$	2.752	2.666	2.752	2.379	2074	2.379	2074	1856	2.580
U (%)	81	83	70	89	93	80	91	78	69

n: number of specimens, U: value of the statistic, $U_{\max} = n(\text{males}) \times n(\text{females})$, $U(\%) = 100 - (U/U_{\max} \times 100)$. Bcl: Bony crest length, Bch: Bony crest height, Bcw: Bony crest width, Bca: Bony crest area, Kca: Keratin crest area, BcCS: Bony crest centroid size, KcCS: Keratin crest centroid size, Sl: Skull length, Sw: Skull.

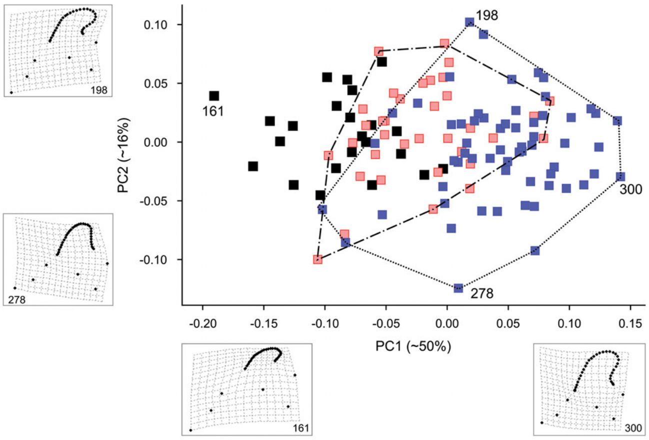

Fig. 9. PCA obtained using the bony crest of the adult males, adult females, and adult *indet*. Blue squares: adult males, red squares: adult females, and black squares: adults *indet*.

initial adults groups using a clustering approach [Gaussian mixture, (Cordeiro-Estrela et al., 2008; Scrucca et al., 2016)]. The clustering is based on size-free values corresponding to the residuals of the PCA applied on the bony crest shape of the adults. The clustering shows that in 72% of the cases, the initial identification can be found (Supplementary Fig. 1, Supplementary Table 1), which validates our initial identification, and shows the independence of our data between the initial sex identification and the measurements used for the further analyses.

The comparison between these predefined groups of males and females using the anatomical measurements shows that the males have larger crests and skulls than the females (Fig. 8), which have been confirmed by the statistical analysis (see in Table 4 that the P-values are always below 0.05). Moreover, the statistic U shows that the keratin crest area (Kca) is the best parameter to distinguish males and females with a U of 93%.

The difference between males and females is also visible in terms of the shape of the bony crest (Fig. 9), as well as for the keratin crest (Fig. 10). The two PCA, respectively, include all the adults having a complete bony crest or a complete keratin crest. As for the anatomical measurements, the females have a smaller crest than the males, but in the case of the bony crest, there is a notable overlap between the sexes, which can complicate the identification of the sexes (Fig. 9). However, the PC1 explains

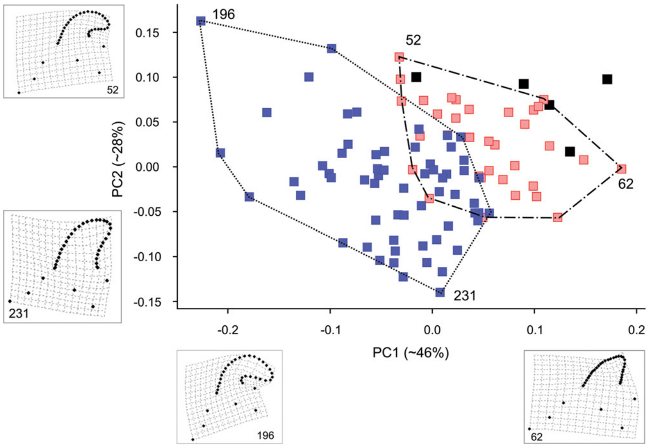

Fig. 10. PCA obtained using the keratin crest of the adult males, adult females, and adults *indet*. Blue squares: adult males, red squares: adult females, and black squares: adults *indet*.

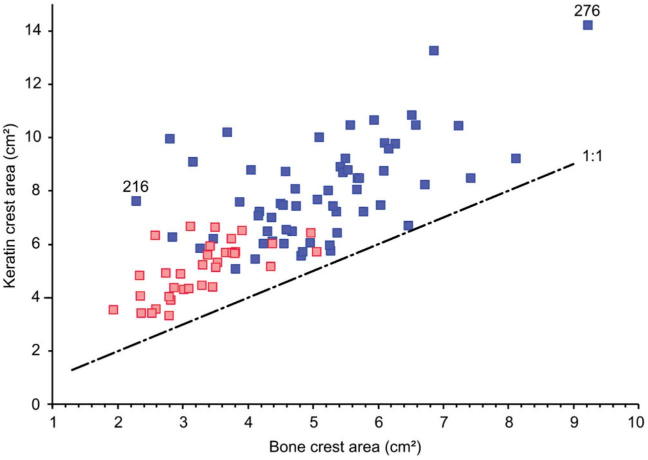

Fig. 11. Bony crest area as a function of the keratin crest area for the adult guinea fowl. Red squares: females, blue squares: males. Black line: linear line of 1:1 corresponding to the cases for which the keratin crest area is the same as than the bony crest area. The specimens 276 and 216 correspond to two specific specimens among the adult males, for which the difference between the bony part and the keratin part of the crest is significantly different.

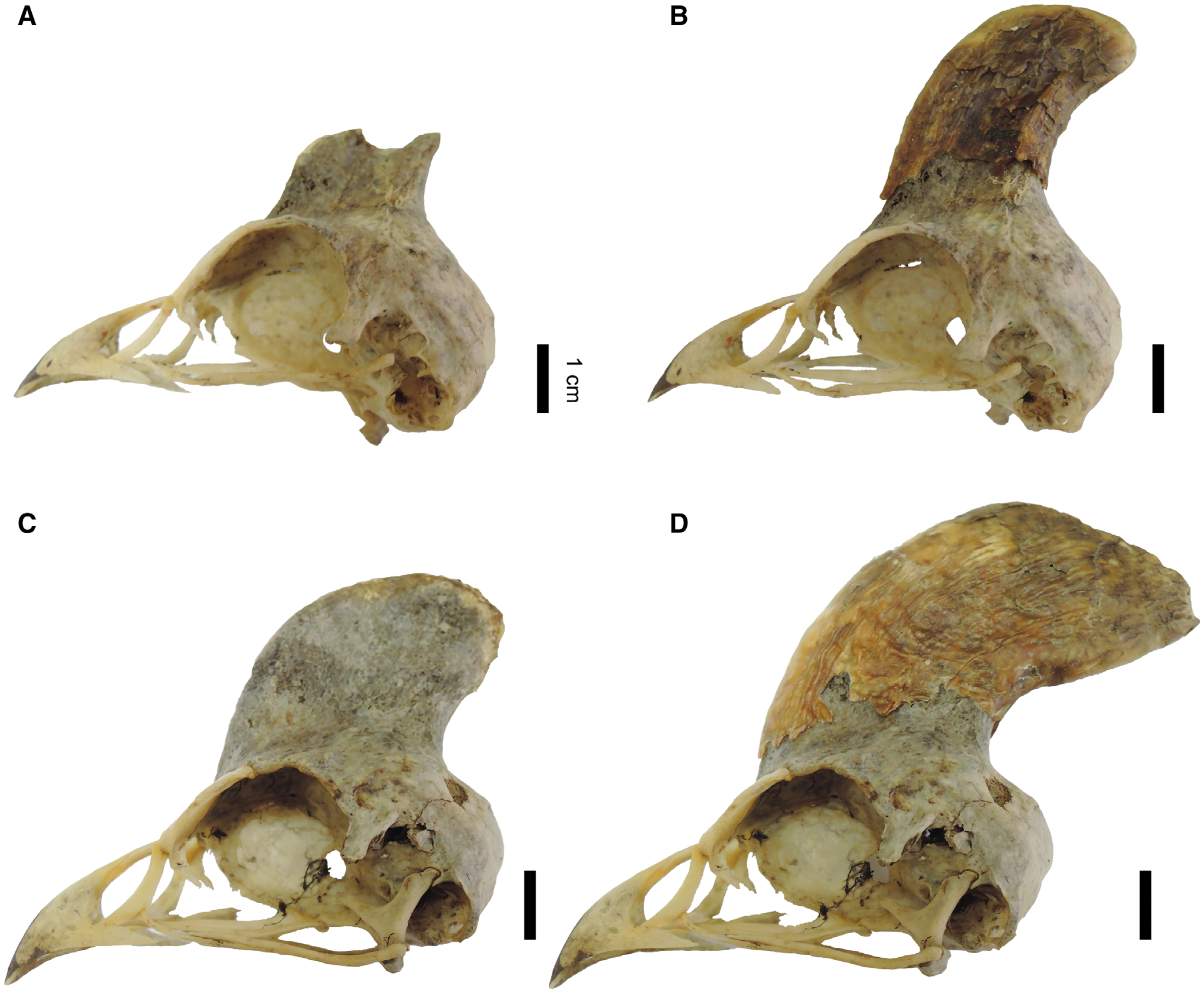

Fig. 12. Comparison of two adult specimens showing the skulls without the keratin covering and with the keratin covering: Specimen 213: (**A**) skull showing the bony crest in lateral view, (**B**) lateral view of the skull with the bony crest covered with its associated keratin crest. Specimen 276: (**C**) skull showing the bony crest in lateral view, (**D**) lateral view of the skull with the bony crest covered with its associated keratin crest.

50% of the variability between the sexes, with the females having bony crests more compact dorsoventrally while the males have taller crests, with a more complex shape including curvature of the posterior part along the dorsoventral axis (Fig. 9). The discrimination between the sexes is much clearer if we consider the keratin crest (Fig. 10). The keratin crest is less developed dorsoventrally and its shape is simpler for the females, which show limited posterior curvature. On the contrary, the males have a well-developed keratin crest, with marked posterior curvature, highlighted by the PC1, which explains around 46% of the variability (Fig. 10). It is noteworthy that the bony crest and the keratin crest of the adults *indet*. (which were secondarily identified as adults based on their fused frontal bones) always overlap with the females (Figs. 9 and 10). This observation concurs with the clustering results, in which 22 adults *indet*. have been identified as females with only one specimen falling among the males (Supplementary Fig. 1, Supplementary Table 1).

Difference between bony crest and keratin crest. Among the adults in our sample, 94 specimens had the keratin crest preserved, which was removed during the preparation of the specimens, but kept with them. For these individuals, it was therefore possible to compare the keratin crest and the actual bony crest. We used the crest area for the comparison. If the Kca approximates the bony crest area (Bca) for the same specimen, we can conclude that the keratin crest is strongly associated with the bony crest. Figure 11 shows that for all the adults considered, the keratin crest is always larger than the bony crest (all the specimens are above the 1:1 line). This is more visible for the males, and specifically for the ones that have a large and well-developed keratin crest. For example, in specimen 276 the keratin crest is 1.5

times the bony crest (Fig. 11 and 12C,D), while in specimen 216 the keratin crest is 3.3 times larger than the bony crest (Fig. 11 and 12A,B). In the latter case, the bony crest is not broken but appears to be laterally compressed making it small in comparison to the well-developed keratin crest.

DISCUSSION

Development of the Crest through the Ontogeny

A bony crest is visible for all our specimens including the youngest individual in our sample (specimen 194). In order to make ontogenetic comparisons, we ranked them using the skull lenght (Sl) as a proxy of the size and age, as proposed by Farke et al. (2013). The largest specimen (specimen 306) has a skull length of 68.2 mm and corresponds to 100% of the skull length. Using this maximum value, we calculated the percentage of the maximum skull length for all the other specimens (Fig. 13). Thus, the youngest specimen (specimen 194) is also the smallest, even though its Sl already corresponds to 84% of the maximal skull length. This highlights the fact that the juveniles sampled were not chicks or early-stage juveniles since, during hunting, only the "larger" individuals were targeted.

We can be more precise and confirm this hypothesis using the work done by Hogg (1978) on chickens. Hogg (1978) studied the timing of the fusion of the cranial sutures and provided a record of when (in terms of number of days after hatching) that these events occurred. Assuming that the timing is similar for the guinea fowl, we can estimate the age of the juveniles in our sample. The careful observation of the skull of specimen 194, using pictures and a 3D micro CT-scan, shows that the parietal and the squamosal bones are unfused (Fig. 4). According to Hogg (1978), the fusion of these bones occurs after 81 postnatal days, allowing us to deduce that the youngest specimen (number 194) in our sample died prior to 81 days post-hatching. Moreover, the suture between the exoccipital and the prootic is fused in this specimen, and according to Hogg (1978), this occurs at 77 postnatal days. Thus, we can deduce that specimen 194 died after 77 days of life, but before reaching 81 days post-hatching (Fig. 13). Furthermore, one of the oldest juvenile specimen in our sample (specimen 187) shows unfused frontal bones but the suture between the frontal and the parietal is completely fused (Fig. 4). Using Hogg's (1978) data, we can deduce that this specimen was older than 98 days but younger than 102 days post-hatching. These estimations confirm that our sample does not include any newly hatched chicks or young juveniles, which limits our deductions for the earliest stages of ontogeny and restricts comparisons with very young crested modern and fossil vertebrates.

Relatively few other vertebrates with bony crests have been studied (Dodson, 1975; Horner and Currie, 1994; Evans, 2010; Brink *et al*., 2011; Farke et al., 2013; Naish and Perron, 2014, Eastick et al., 2019). Farke et al. (2013) proposed a summary, showing the growth stage when the bony crest begins to form in different vertebrates (specimens indicated with a star in the Fig. 13). Farke et al. (2013) show that the bony crest starts its development at different stages depending on the group studied: A crest is visible in the early juvenile stages of *Parasaurolophus* (25% of the maximum skull size), at about 50% of the ontogeny for *Corythosaurus* (50% of the maximum skull size), and at a subadult stage of growth of modern cassowaries (83% of the maximum skull size) (Fig. 13). In the case of the modern guinea fowl studied here, the earliest stage when a crest is visible is for a size around 84% of the maximal skull size, which is significantly later than for the two dinosaur groups previously studied while among cassowaries the crest began developing when the skull size reached between 65 and 83% adult size (Dodson, 1975). These findings among modern birds suggest that crest development begins later than among the dinosaurs. Farke et al. (2013) suggested that this difference could be explained by the fact that it is generally held that dinosaurs reached sexual maturity prior to skeletal maturity (Erickson et al., 2007; Lee and Werning, 2008), which contrasts with neornithines birds that generally reach sexual maturity after attaining skeletal maturity (Dodson, 1975). We propose that the crest development in the guinea fowl is like that of cassowaries, that is, the development of the bony crest begins during late ontogeny, prior to the attainment of sexual maturity, and that by the time sexual maturity is attained the bony crest would be well-developed.

The bony crest of the guinea fowl is formed from the two frontal bones (Fig. 4), and it appears that this is also the case for cassowaries (Naish and Perron, 2016). However, the bony crest of modern cassowaries extends anteriorly to the base of the rostrum and fuses with the posterodorsal ramus of the premaxilla (Naish and Perron, 2016). In the helmeted guinea fowl, we found that the bony crest is strictly restricted only to the frontal bones. Among other vertebrates with bony crests, *Pteranodon*'s crest is also formed by the frontals, but in this case, the structure extended upward and/or backward from the posterior end of the skull (Bennett, 1992). Given that, pterosaurs probably attained sexual maturity prior to reaching skeletal maturity (Chinsamy *et al*. 2009), we propose that the development of the crest probably began early during ontogeny (as proposed for dinosaurs). Among the dinosaurs, oviraptorosaurs and *Parasaurolophus* have crests mainly formed by the nasals, but some oviraptorids (e.g., *Citipati*, *Nemegtomaia*, and *Rinchenia*) also incorporate the frontals into their crest (Farke et al., 2013; Currie et al., 2016; Funston et al., 2018). Thus, it is apparent that the actual formation and development of the crest among extinct and extant vertebrates is highly variable.

A bony crest is present for all the juvenile guinea fowl specimens studied here, and we observe a large variation in terms of the crest size and shape during ontogeny. A positive allometric relationship is observed between the isometric size of the skull and the isometric size of the bony crest (Figs. 6 and 13). This result shows that the youngest specimens, which have the shortest Sls, have very small, dorsoventrally compressed crests with simple shapes. On the other hand, the subadults have the longest skull lengths, and they have well-developed crests that are almost as large as the ones observed for the adult specimens. This is different to the observations made on cassowaries by Dodson (1975) in which he noted that the casque is absent at the hatchling stage and during the first 2 years, and only begins to form once the skull length reaches 135–150 mm (i.e., about 65%–85% of adult size). He also found that during the early stages of development, the skull height shows a negative allometry with the skull length, but thereafter once the casque begins to develop, the skull height becomes strongly

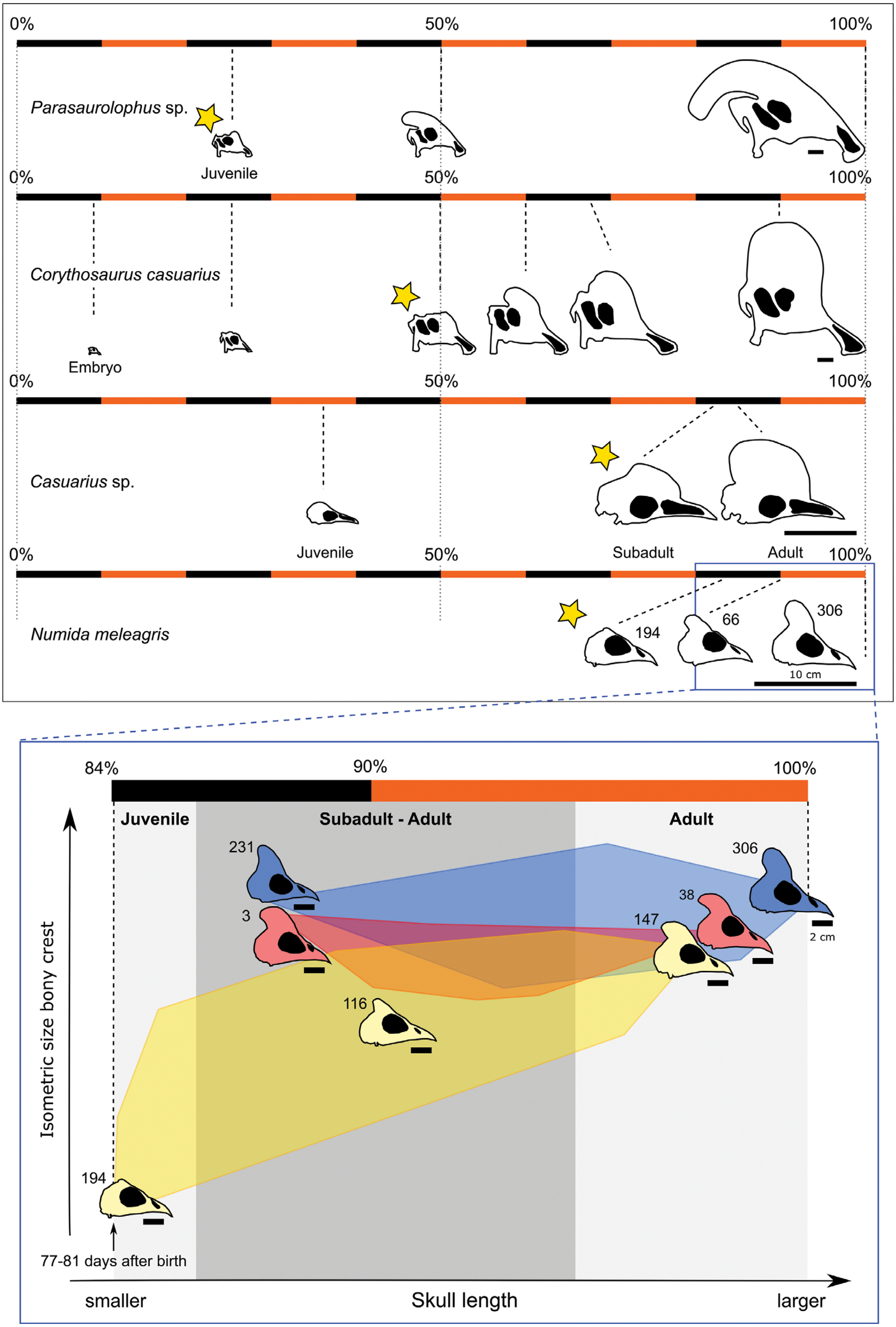

Fig. 13. Legend on next page.

positively allometric with the skull length (Dodson, 1975; Naish and Perron, 2016). Although our study material did not contain any hatchlings or chicks, we also observed similar allometric trends when the guinea fowl skulls reached around 80% of the maximum size. Among cassowaries, the positive allometry starts only after 2 years, while our data suggest that a positive allometry is already visible at 77 days after hatching (Fig. 13). This variation in timing could be explained by the body size difference between cassowaries and guinea fowls, and it may be that the former require more time to reach their adult size than the latter.

Sex Identification

Our study of the helmeted guinea fowl has permitted a documentation of the crest development through the ontogeny and has also allowed an assessment of the variation evident between males and females. Our study shows that statistically, males have larger skulls with larger, complex-shaped bony and keratin crests than females (Table 4, Figs. 8, 9, and 10). However, there is considerable overlap between the sexes, which complicates the distinction between the two groups (Figs. 8, 9, and 13). This problem has already been pointed out by Prinsloo et al. (2005), who used the helmet height and width, the wattles length and width, the ceres development and the body mass, to study the potential sexual dimorphism in the guinea fowl. Indeed, even with discriminant analysis, employing all these parameters, they obtain only 74% of correct sex prediction. Their results can be explained by their choice of parameters, which pose a problem of repeatability and objectivity, for example, some of the characteristics are soft tissues such as the wattles, or the ceres, which are not always preserved. Moreover, the development of the wart-like cartilaginous structure of the ceres is a rather subjective character, which they scored out of 100 with no real defining limits. Finally, some of their measurements are not directly linked to an anatomical point or any maximal extremities (such as the helmet width) making the repeatability impossible. For these reasons, new parameters were defined and used for the present research. Another issue worth mentioning is that Prinsloo et al. (2005) used a large sample ($n = 579$ adults) of birds that were obtained from a large area between the towns of Heibron, Petrus Steyn, Frankfort, and Hoogte, which may have introduced population differences, especially considering that Crowe (1979) has previously suggested that the variability of the crest could be linked to environmental factors.

Our results show that the most discriminant character between the sexes is the keratin crest area (Kca), which permits correct sex identification in 93% of the cases. However, if the keratin crest is not preserved, the bony crest area (Bca) is also a useful discriminant parameter between males and females (89%) (Fig. 8 and Table 4). The crest areas are the best parameters since contrary to the other measurements, such as the crest length or height, they consider the shape of the crest. The significance of the crest shape is highlighted by the results obtained from the PCA assessment of the keratin crest (Fig. 10). The results show a clear discrimination between males, which have a well-developed, dorsally curved crest, and females which have a simpler, less curved keratin crest. Our results caution against the use of simple anatomical measurements because they do not necessarily reflect the real variation between groups.

The differences observed between males and females could be interpreted as a sexual dimorphism with males having a larger, more complex crest than females. However, the large overlap of the data (Figs. 8 and 9) suggests that both sexes have well-developed crests. Perhaps the size and shape complexity of the crest is an indicator of individual fitness, and it may be that this trait corresponds to mutual sexual selection. However, it should be noted that the groups of males and females were initially identified using external anatomical characteristics (which are rather subjective and not totally discriminant). Moreover, some of the criteria used for this initial identification also involved the crest, thus making them not independent. However, our secondary sexual identification using a clustering approach permitted the validation of our initial groups by up to 72%, using an independent criterion. However, since the recovery is not perfect between the initial sex groupings and those identified by the clustering, it is possible that some initial identification errors may have crept in. To resolve this problem, future studies should ensure an unambiguous sex identification method, such as gonad identification or genetic assessment.

It is difficult to explain the growth process, which drives the differences observed in the size and shape of the crest between the sexes since the precise age at death is unknown. However, Figure 13 shows that, after the rapid growth of the skull and the crest during the juvenile and subadult stages, the growth of the crest slows down drastically at the adult stage, while the skull continues to grow. The difference between males and females could maybe be explained by differences in the rate of growth between the sexes during the ontogeny, but this cannot be verified.

Adults *Indet.* and Secondary Identification

Using the criteria of fusion between the two frontal bones, we were able to secondarily identify juveniles and adults. During this process, we found that some previously identified juveniles were adults, which we denoted as adults *indet*. As explained above, this incorrect initial attribution is due to the utilization of unreliable anatomical characteristics (Prinsloo et al., 2005). It is interesting to note that almost all the adults *indet.* fall among females or between the juveniles and the females (Figs. 6 and 7). The same observation was made using the clustering results: among the 23 adults *indet.*, the clustering approach attributed 22 specimens to females and only one as male (Supplementary Fig. 1, Supplementary Table 1).

Fig. 13 Cranial growth series for *Parasaurolophus*, *Corythosaurus*, *Casuarius*, and *Numida meleagris*. Top part—Black and orange scale: percentage of maximum reported skull length in increments of 10%, yellow stars: approximative skull size at which ornamentation initially appears [Modified from Evans, 2010]. Blue frame at the bottom—Variation in the isometric size of the bony crest through the time (estimated from the skull size). The skull length could be interpreted as a proxy for the ontogenetic stage. Yellow area: juvenile specimens, red area: female specimens, and blue area: male specimens. The numbers next to the skull silhouettes correspond to the specimen numbers.

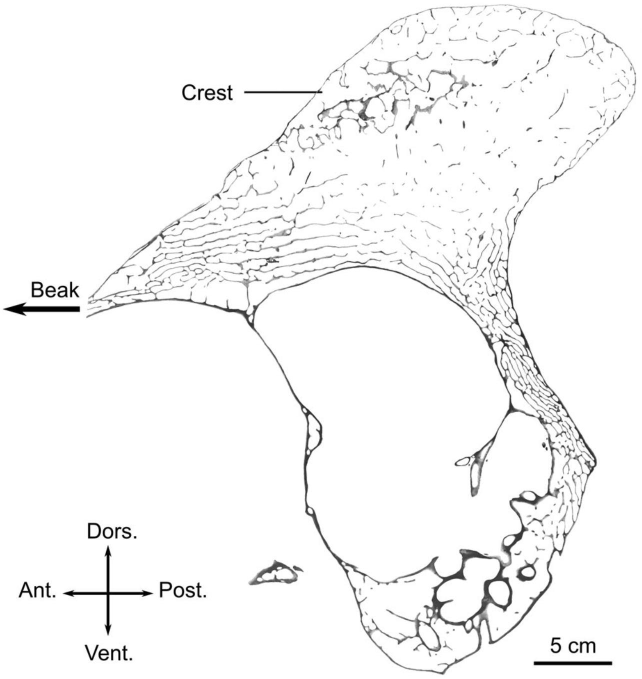

Fig. 14. Bony crest internal structure obtained with a longitudinal thin section, showing the very porous structure of the crest.

Difference between Bony Crest and Keratin Crest

Figures 11 and 12 highlight the fact that the keratin crest is always larger than the underlying bone. For example, specimen 276 has a keratin crest 1.5 times larger than its bony crest, while in specimen 216 the keratin cap is three times that of the bony crest (Figs. 11 and 12, and Supplementary Table 1). Thus, the size and the shape of the bony crests observed in some fossil vertebrates could underestimate the actual size and shape of the keratinous covering during life, as already shown for claws or horns (Brown, 2017).

In most of the cases, the keratin crest is slightly larger than the bony crest, but for some specimens such as specimen 216, the much larger overlying keratin can be explained by the lateral compression of the bony crest in the dorsal region (Fig. 12A). Thus, assessing only the small size of the bony crest could lead to the misidentification of the specimen as a juvenile, but considering the structure of the bony crest with the fused frontals and the large keratin cap clearly leads identifies the individual as an adult. This kind of reduction in the size of the bone crest has never been reported previously for any other vertebrate. The apparent reduction in the size of bony crest during late ontogeny may be similar to the horn resorption evident in old ceratopsians (Scannella and Horner, 2010).

Function of the Crest

Among crested birds and other vertebrates, several interpretations and functions have been previously proposed to explain the development of cranial crests. For example, in some chameleons, the crest serves as a site for muscle attachment (Measey et al., 2009), but this is not the case for the guinea fowl. Among pterosaurs or cassowaries, the cranial crests have been linked to thermoregulation (Kellner and Campos, 2002; Eastick et al., 2019), but Crowe (1979) showed that this is clearly not the case for the guinea fowl. Could the guinea fowl crest be involved in communication? The helmeted guinea fowl makes a distinctive cackle used for intraspecific recognition as well as the protection of the group (Crowe et al., 1986). For some modern birds such as the cassowary, it has been suggested that the crest is a resonance chamber for low-frequency communication (Naish and Perron, 2016), while it has also been suggested facilitating the detection of infrasonic calls (Mack and Jones, 2003). Mack and Jones (2003) proposed that the size and the shape of the crest are intrinsically linked to vocal signaling and the advertising of fitness. It is quite possible that the crest of the guinea fowl could be involved in a similar kind of communication, but this is beyond the scope of the current study. The keratin sheathed casques of cassowaries are thought to function as a tool to move debris on the forest floor (Folch, 1992), as a foliage deflector, for knocking hanging branches to dislodge fruit (Naish and Perron, 2016), for cranial protection when the birds charge through undergrowth (Crome and Moore, 1988) or during intraspecific aggression (Naish and Perron, 2016). Naish and Perron (2016) countered all these hypotheses by showing that the internal structure of the cassowary crest is relatively fragile and there are no (or very few) observational studies that support those potential functions. It should be noted that like the cassowary, the guinea fowl also has a similarly porous and fragile crest, thus also limiting its functionality (Fig. 14).

As with other crested vertebrates, the guinea fowl crest is quite likely to have functioned in species recognition (Horner et al., 2000) and sexual dimorphism (Bennett, 1992; Hone et al., 2012; Naish and Perron, 2016). Crowe (1979, 2000) examined different species of guinea fowls and showed that they each had different crest size, shape, and color, as well as different colors of feathers, head skin, and wattles, which may have been important for species recognition. However, he also pointed out that the crest characteristics were probably linked to specific environmental parameters, and that among particular species, many variations occurred. Since our study sample derives from the same location and population, we propose that the observed variation in the crest size and shape could be explained by a sexual dimorphism, and that as suggested for cassowaries by Naish and Perron (2016) the crest could be an indicator of fitness. Since both sexes show a relatively well-developed crest, we propose that the guinea fowl crest is linked to a mutual sexual selection (Hone et al., 2012; Naish and Perron, 2016).

Thus, although the exact function of the crest of the helmeted guinea fowl is unclear, and further studies, as well as field observations, are needed, we propose that it is quite likely used as an acoustic tool, which will be explored further in a future study, and that it is involved in mutual sexual selection.

CONCLUSIONS

This study of a large sample of skulls of the helmeted guinea fowl including juveniles and adults has permitted the following deductions:

1. Despite the absence of newly hatched individuals, the juveniles in our study are statistically smaller than adults for all the anatomical measurements.

2. Bony crest height (Bch) appears to be the most discriminant anatomical measurement (permitting a 94% accuracy) between juveniles and adults.
3. The bony crest in guinea fowls begins developing from the frontal bones of the skull from juvenile stages onward.
4. Among adults, females are statistically smaller than males for all the anatomical measurements, and the most discriminant measure is the keratin crest area (Kca) with 93% correct attribution.
5. Juveniles have smaller, simpler crest shapes as compared to the adults, while among the adults, males have more well-developed keratin crests with highly variable shapes.
6. Among adults, the keratin crest does not usually mimic the size and shape of the underlying bony crest. In some cases, the keratin crest is three times larger than the bony crest. This finding has direct implications for interpretations and reconstructions of fossilized crested vertebrates.
7. The function of the helmeted guinea fowl crest remains unclear and requires further investigations, but it seems likely that it played a role in acoustics and mutual sexual selection.

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